Microtubule stiffening by doublecortin-domain protein ZYG-8 contributes to spindle orientation during *C. elegans* zygote division

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ABSTRACT

To address the role of microtubule flexural rigidity in spindle positioning during cell division, we used the *Caenorhabditis elegans* zygote. We showed that ZYG-8^{DCLK1}, its unique Doublecortin-family member, rigidifies microtubules, as evidenced by more bent microtubules when targeting this protein. Microtubules also exhibited larger maximal curvatures and increased tortuosity. Functionally, we observed increased amplitudes of anaphase spindle-pole oscillations following *zyg-8(RNAi)* and even greater in *zyg-8(or484ts)* mutants preventing microtubule binding. Interestingly, the mutants exhibited incorrect final spindle position and orientation. Overly soft microtubules impaired spindle centring force, upsetting the pulling versus pushing force balance. We strengthened these findings by depleting PTL-1^{Tau}, a protein that rigidifies microtubules. In conclusion, sufficient microtubule rigidity is essential during mitosis to ensure effective cortical pushing, thereby accurately orienting the spindle and guaranteeing correct daughter cell fates. Microtubule rigidity is likely controlled in human mitoses as well, consistent with reported DCLK1 and Tau deregulations in various cancers.

INTRODUCTION

Microtubules are semi-rigid polymers that form a crucial component of the cell cytoskeleton alongside actin and intermediate filaments. They perform essential functions with various molecular players, including cell shaping (Matis, 2020; Rizzelli et al., 2020), intracellular trafficking (Burute and Kapitein, 2019), and cell migration (Garcin and Straube, 2019). They also play major roles in cell division; for example, spindle microtubules assemble into the mitotic spindle and segregate chromosomes, while astral microtubules help position the spindle (di Pietro et al., 2016; Forth and Kapoor, 2017; Kotak, 2019; Lechler and Mapelli, 2021; McNally, 2013; Thomas et al., 2017; van Leen et al., 2020). Along a structural line, each microtubule comprises protofilaments that are linear chains of tubulin dimers added or removed dynamically. Microtubule dynamic instability, characterised by stochastic transitions between growing and shrinking phases, is regulated *in vivo* by numerous Microtubule Associated Proteins (MAPs) (Akhmanova and Kapitein, 2022; Goodson and Jonasson, 2018; Gudimchuk and McIntosh, 2021). The mechanical properties of microtubules, particularly their flexural rigidity—i.e., their resistance to bending—have received comparatively less attention. Notably, microtubules exhibit the highest flexural rigidity among cytoskeletal filaments (Gittes et al., 1993). While *in vitro* reconstituted microtubules are bent at the scale of a few millimetres (Felgner et al., 1996; Gittes et al., 1993; Kurachi et al., 1995; Mickey and Howard, 1995), *in vivo* observations revealed that microtubules appear bent at the micrometre scale (Brangwynne et al., 2006; Hawkins et al., 2010; Zhang et al., 2017). On the one hand, high microtubule rigidity is necessary for efficient intracellular trafficking (Balabanian et al., 2017) or pushing against obstacles, such as chromosome arms producing the polar ejection force (Maiato et al., 2017; Schneider et al., 2022). On the other hand, microtubules must also be flexible enough to bend and adapt to the shape of the cell as they navigate through the crowded cytoplasm (Arjona et al., 2023). It calls for regulating microtubule flexural rigidity *in vivo* to support its various biological functions (Hawkins et al., 2010).

Several mechanisms have been proposed to achieve such a regulation, with two key families of proteins identified: the Doublecortin family, including DCX and DCLK1, and the MAP2/MAP4/Tau superfamily (Felgner et al., 1997; Kurachi et al., 1995; Mickey and Howard, 1995; Nishida et al., 2023). For instance, the simultaneous knockdown of DCX and DCLK1 in rat neurons led to more curved microtubules at the growing axon tip (Jean et al., 2012). Doublecortin family proteins associate with microtubules through their doublecortin domains. Notably, they bind between the microtubule protofilaments at the vertices of four tubulin dimers, contributing to the lateral coupling of adjacent protofilaments (Fourniol et al., 2010; Moores et al., 2004). This binding likely hinders protofilament sliding, making the microtubule stiffer (Pampaloni et al., 2006; Sui and Downing, 2010). Along a similar line, Tau-deficient axons have been shown to exhibit curled microtubules (Hahn et al., 2021). Tau family proteins bind longitudinally along the outer ridges of microtubule protofilaments. It would strengthen the longitudinal contacts between tubulin heterodimers within a protofilament, straightening them (Kadavath et al., 2015; Kellogg et al., 2018). Importantly, both protein families also contribute to the regulation of microtubule dynamics. *In vivo* studies indicate that these proteins stabilise microtubules and promotes their growth (Hahn et al., 2021; Srayko et al., 2005). They may also reduce their dynamics by decreasing catastrophe frequency, depolymerisation and nucleation rates (Bunker et al., 2004; Dema et al., 2024; Srayko et al., 2005). While the impacts of controlling microtubule rigidity have primarily been

studied in neurons, microtubule-rigidity contribution during cell division also warrants investigations.

To explore this potential role, we reviewed previous cell-division studies targeting proteins from the Doublecortin and Tau families. Interestingly, targeting a protein from the Doublecortin family resulted in several cell division perturbations, including mispositioning of the mitotic spindle, defects in the formation and integrity of meiotic or mitotic spindles, and impaired mitotic progression (Couillard-Despres et al., 2004; Czajkowski et al., 2024; Gönczy et al., 2001; Kaplan and Reiner, 2011; Li et al., 2023; Shu et al., 2006; Vreugdenhil et al., 2007). During neurogenesis, some of these studies led to incorrect cell fate determination for neural progenitors (Shu et al., 2006; Vreugdenhil et al., 2007). Additionally, the deregulation of Tau (whether through mutation or overexpression) resulted in mitotic defects, such as issues with spindle formation, aneuploidy and chromosome misalignment (Bougé and Parmentier, 2016; Rossi et al., 2008). Thus, targeting Doublecortin and Tau family proteins can lead to a wide range of meiotic and mitotic disturbances, although the underlying mechanisms remain unclear. Proposed explanations include perturbations in microtubule stability, and in molecular motor activity or recruitment (Czajkowski et al., 2024; Gönczy et al., 2001; Kaplan and Reiner, 2011; Li et al., 2023; Liu et al., 2012; Shu et al., 2006; Vreugdenhil et al., 2007). Whether the underlying mechanism involves microtubule rigidity is yet to be investigated in dedicated studies.

Along this line, we and others have proposed that microtubule buckling—i.e., bending under compressive forces—contributes to the precise and stable maintenance of the mitotic spindle at the cell centre during the metaphase of the *Caenorhabditis elegans* zygote division (Bouvrais et al., 2021; Garzon-Coral et al., 2016; Howard, 2006; Pécréaux et al., 2016). While this division is asymmetric, the mitotic spindle initially orients along the polarity axis and centres within the embryo during prophase. It is then maintained at the cell centre during metaphase and is finally displaced toward the posterior side, mostly during anaphase (Oegema and Hyman, 2006). The correct positioning and orientation of the spindle during asymmetric divisions are critical for cell fate determination and, more broadly, for the organism's development (Lechler and Mapelli, 2021). These processes primarily depend on dynamic astral microtubules emanating from the centrosomes, which can transmit or generate three types of forces (Howard and Garzon-Coral, 2017; Kotak, 2019; McNally, 2013): first, cortical pulling forces mediated by dynein motors anchored at the cell cortex interacting with shrinking microtubules (Grill and Hyman, 2005; Kozlowski et al., 2007; Nguyen-Ngoc et al., 2007); second, cytoplasmic pulling forces involving motile dynein interacting with organelles within the cytoplasm (Kimura and Kimura, 2011); and third, cortical pushing forces generated by microtubules that grow against the cell cortex (Garzon-Coral et al., 2016; Howard, 2006; Pécréaux et al., 2016). Given that pushing forces rely on stiff microtubules, we proposed that their rigidity be controlled to ensure the proper choreography of the spindle during *C. elegans* division. Specifically, we envisioned that sufficiently high microtubule rigidity may be required to balance elevated cortical pulling forces during anaphase, the latter participating in spindle elongation and posterior displacement.

To address the potential role of microtubule flexural rigidity in cell division, we utilised the *C. elegans* embryo, a well-established model of asymmetric cell division with low functional redundancy. Notably, *zyg-8* and *ptl-1* are the only members of the Doublecortin and Tau families, respectively (Goedert et al., 1996; McDermott et al., 1996; Reiner et al., 2006). Mutations in doublecortin or kinase domains of *zyg-8* have been shown to induce incorrect

spindle final positioning and orientation (Gönczy et al., 2001). Moreover, ZYG-8 depletion or mutation caused defective meiotic spindles, either longer and bent (still-bipolar) or multipolar (Czajkowski et al., 2024; Li et al., 2023). Overall, it led us to target *zyg-8* to manipulate microtubule rigidity. We employed three complementary genetic perturbations: reducing ZYG-8 expression via RNA interference, overexpressing it and hindering its microtubule binding using a *zyg-8(or484ts)* mutant (Bellanger et al., 2007). We measured the distributions of microtubule local curvatures to assess variations in microtubule rigidity alongside examining spindle positioning through various biophysical techniques to get corresponding division phenotypes. Our findings demonstrate that ZYG-8 is needed to maintain the correct spindle orientation during mitosis, primarily by permitting high centring forces that depend on the microtubule flexural rigidity. Interestingly, the depletion of PTL-1 through RNAi exhibited a comparable but milder effect on spindle choreography. Our study highlights the need for sufficient microtubule rigidity for proper mitotic spindle positioning and orientation during *C. elegans* zygote division.

RESULTS

1- ZYG-8 likely regulates microtubule flexural rigidity.

As ZYG-8^{DCLK1} was suggested to control microtubule curvatures in neurons, we set out to validate its role in the one-cell embryo. By immunofluorescence, ZYG-8 was localised at the spindle and its poles (Gönczy et al., 2001). To test its association with the astral microtubules, we generated three strains by CRISPR-Cas9: one with fluorescently-labelled ZYG-8 endogenously expressed, mNG::ZYG-8; a second with *zyg-8* tagged at the locus with three OLLAS tags; and a third with an integrated transgene that overexpresses *zyg-8* (*pPie-1*::mNG::3*OLLAS::ZYG-8) on top of the endogenous copy (Table S1). During the first embryonic division of endogenously expressed and overexpressed *zyg-8* strains, ZYG-8 significantly co-localised with tubulin on astral and spindle microtubules (Figure S1, Method M5). This localisation was consistent with its potential role in regulating microtubule flexural rigidity.

To vary the amount of ZYG-8 in the embryo, we altered its expression level by performing RNAi against *zyg-8* or by overexpressing it. Western blot analysis of worm lysates showed decreased or increased OLLAS levels, respectively (Figure S2A-B, Method M6). Additionally, we found corresponding changes in mNG::ZYG-8 fluorescence on both spindle and astral microtubules in the zygotes (Figure S2C-D, Method M6). These results provided an effective means to assess its role in controlling microtubule rigidity.

Next, we explored ZYG-8 potential role in regulating microtubule flexural rigidity in the nematode zygote. To do this, we examined the shapes of the astral microtubules as a proxy similar to previous work in the axonal growth cone (Jean et al., 2012). We imaged the microtubules by immunostaining in fixed embryos using a confocal super-resolved microscope. We observed more bent microtubules in the RNAi-treated embryos (Figure 1C, G compared to A, E). To further test the effect seen by RNAi, we studied the thermosensitive mutant, *zyg-8(or484ts)*, which carries a mutation in the first doublecortin domain (Gönczy et al., 2001; Wood et al., 1980). This mutant has previously been reported to prevent ZYG-8 binding to microtubules when incubated at the restrictive temperature, 25°C, for more than 12 hours (Bellanger et al., 2007). In the mutants, we observed even larger curvatures along

the microtubules at the restrictive temperature (Method M3, Figure 1 D, H compared to B, F). During anaphase, we also noticed fragmented microtubules within the cytoplasm of some mutant embryos (Figure 1H). Taken together, the changes in microtubule shapes upon targeting *zyg-8* suggested a role in regulating microtubule rigidity.

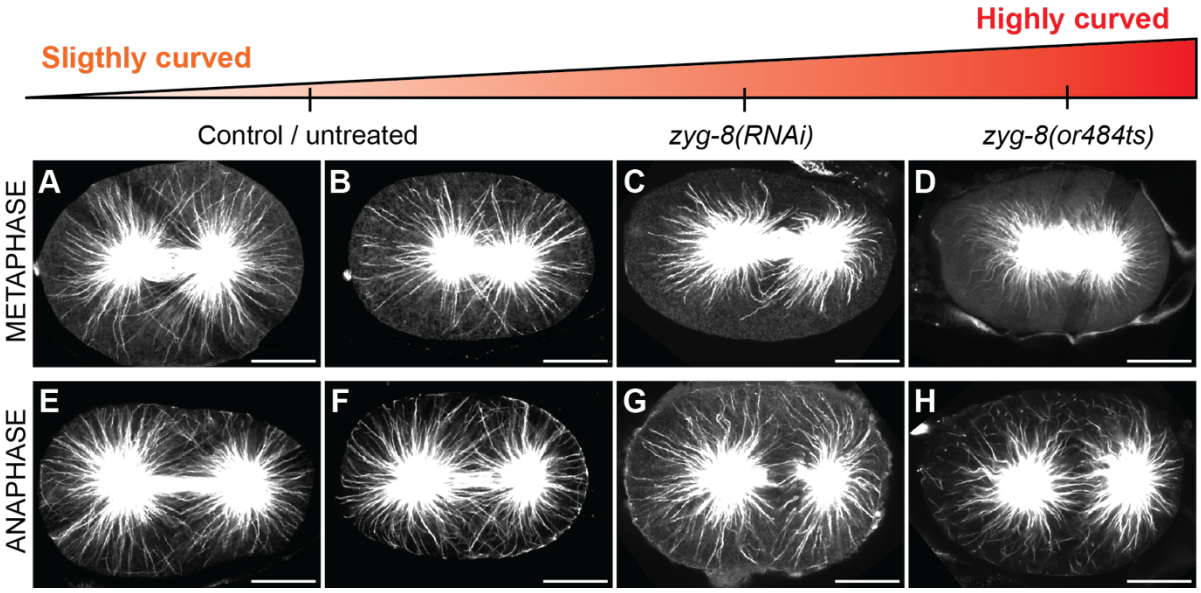

Figure 1: Astral microtubules appear more curved upon targeting *zyg-8*.
(**A-H**) The deconvolved confocal images of fixed embryos with α-tubulin immunostaining reveal microtubule shapes during (A-D) metaphase or (E-H) anaphase in various conditions: (A, E) control embryos, (B, F) untreated embryos at the restrictive temperature, (C, G) *zyg-8(RNAi)* treated embryos, and (D, H) *zyg-8(or484ts)* mutant embryos at the restrictive temperature (Methods M3 and M4). The scale bars represent 10 μm.

To go beyond this visual inspection, we set out to quantify the curvature distribution of the microtubules. Indeed, measuring microtubule rigidity in *C. elegans* embryos cannot be performed using usual *in vitro* methods (e.g. thermal fluctuations, optical trap/tweezers, calibrated flow) (Felgner et al., 1997; Felgner et al., 1996; Gittes et al., 1993; Kikumoto et al., 2006; Kurz and Williams Jr, 1995; Mickey and Howard, 1995; Venier et al., 1994). As an alternative, comparing the distributions of microtubule local curvatures has been previously employed (Bicek et al., 2009; Blob et al., 2024; Zhang et al., 2017). Assuming that the forces applied to the microtubule network are similar between the two studied conditions, a distribution displaying a larger proportion of highly curved microtubules would reflect a lower microtubule rigidity. It, however, requires segmenting the microtubules. The challenge lies in the faint and uneven labelling of microtubules. To tackle it, we assembled a dedicated image processing pipeline in five steps. It allowed us to segment the astral microtubules and then calculate their local curvatures on a pixel-by-pixel basis using a three-point method (Figure S3, Method M7). By generating the averaged curvature distribution for each condition, we observed a slight increase in the proportion of high local curvatures following *zyg-8(RNAi)* treatment (Figures 2A-B & S4A-B). The local curvature distribution of *zyg-8(or484ts)* mutants at the restrictive temperature was significantly different from that of untreated embryos. It showed a more pronounced shift toward higher curvatures than the RNAi treatment (Figures 2C-D & S4C-F). We then focussed on the 95th percentile curvature per microtubule as an indicator of the highest curvature. By measuring the median per z-projected stack of the highest curvatures, we found mild increases for *zyg-8(RNAi)* and large increases in the mutants

(Figure 2E-F). Investigating microtubule curvatures suggested that microtubules were less rigid upon *zyg-8(RNAi)* and even softer in *zyg-8(or484ts)* mutants at the restrictive temperature.

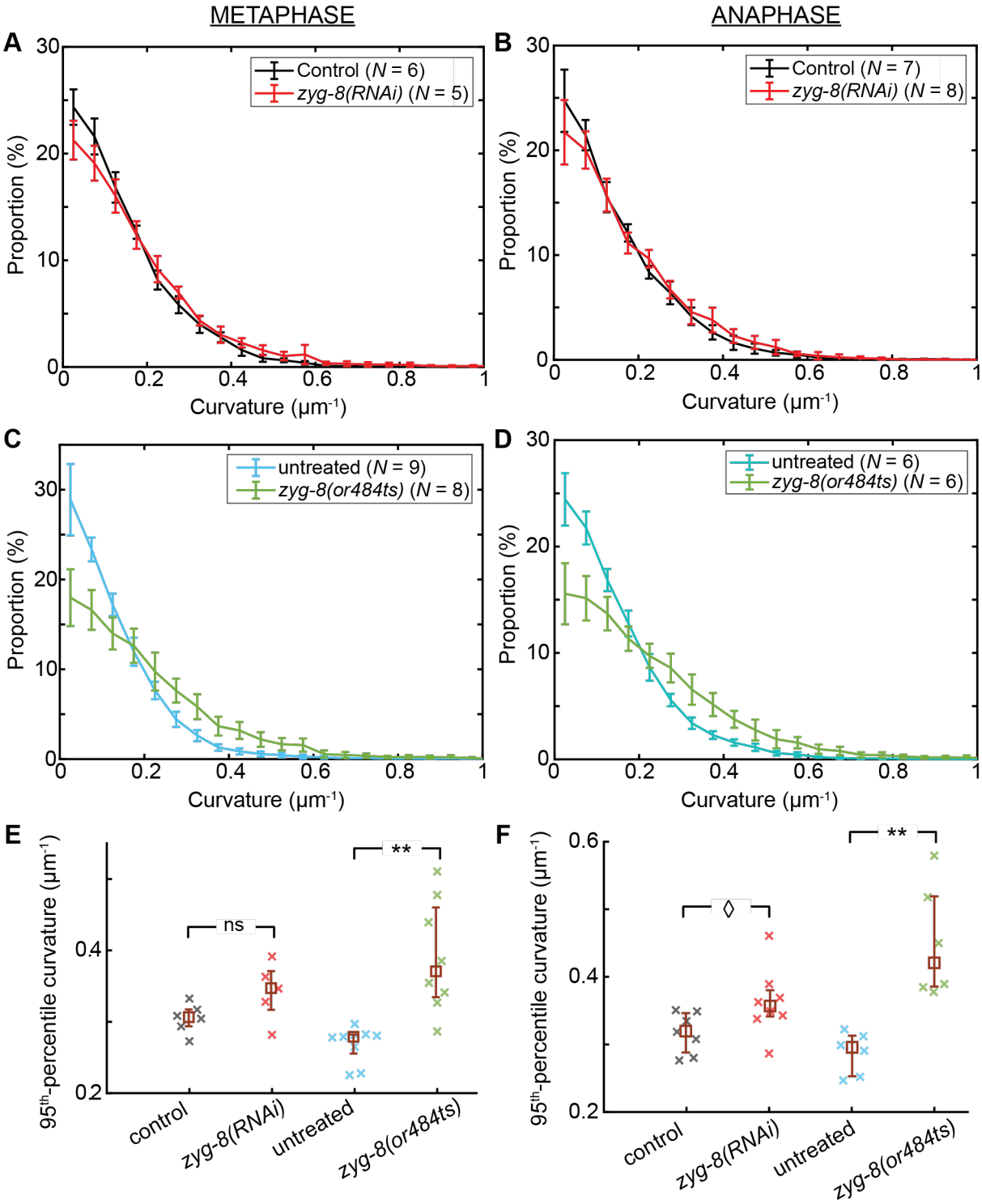

Figure 2: The proportion and magnitude of microtubule curvatures are increased upon targeting *zyg-8*.

(**A-B**) Averaged and normalised distributions of astral microtubule local curvatures of (red) *zyg-8(RNAi)* treated embryos and (black) control embryos: (A) during metaphase with (red) $N = 5$ z-projected stacks and (black) $N = 6$ z-projected stacks (Kolmogorov–Smirnov test: $p = 0.36$, Method M14); and (B) during anaphase with (red) $N = 8$ z-projected stacks and (black) $N = 7$ z-projected stacks (Kolmogorov–Smirnov

test: p = 0.36, Method M14). **(C-D)** Averaged and normalised distributions of astral microtubule local curvatures of (light green) *zyg-8(or484ts)* mutants and (light blue) untreated embryos, both at the restrictive temperature: (C) during metaphase with (light green) N = 8 z-projected stacks and (light blue) N = 9 z-projected stacks (Kolmogorov–Smirnov test: p = 0.03, Method M14); and (D) during anaphase with (light green) N = 6 z-projected stacks and (light blue) N = 6 z-projected stacks (Kolmogorov–Smirnov test: p = 0.014, Method M14). (**E-F**) Medians per z-projected stacks of the highest curvatures (measured as 95th percentile curvature): (E) during metaphase (data are the same as for (A, C)) and (F) during anaphase (data are the same as for (B, D)). Embryos were fixed, immuno-stained for α-tubulin, and local curvatures were extracted (Methods M3, M4, M7). The brown squares and error bars correspond to the medians and the quartiles (Method M14).

We sought a complementary quantification of microtubule shape deformation that does not depend on curvature computation. Indeed, the latter may be sensitive to the microtubule coordinate sampling. We proposed characterising the astral microtubules' tortuosity (Figure 3A) (Nishida et al., 2023). Interestingly, we observed a significantly greater tortuosity following *zyg-8(RNAi)* treatment. It was even more pronounced in *zyg-8(or484ts)* mutants at the restrictive temperature (Figure 3B-C). Taken together, by studying astral microtubule shapes, we found that ZYG-8 controls microtubule curvature and tortuosity, suggesting that ZYG-8 regulates microtubule flexural rigidity.

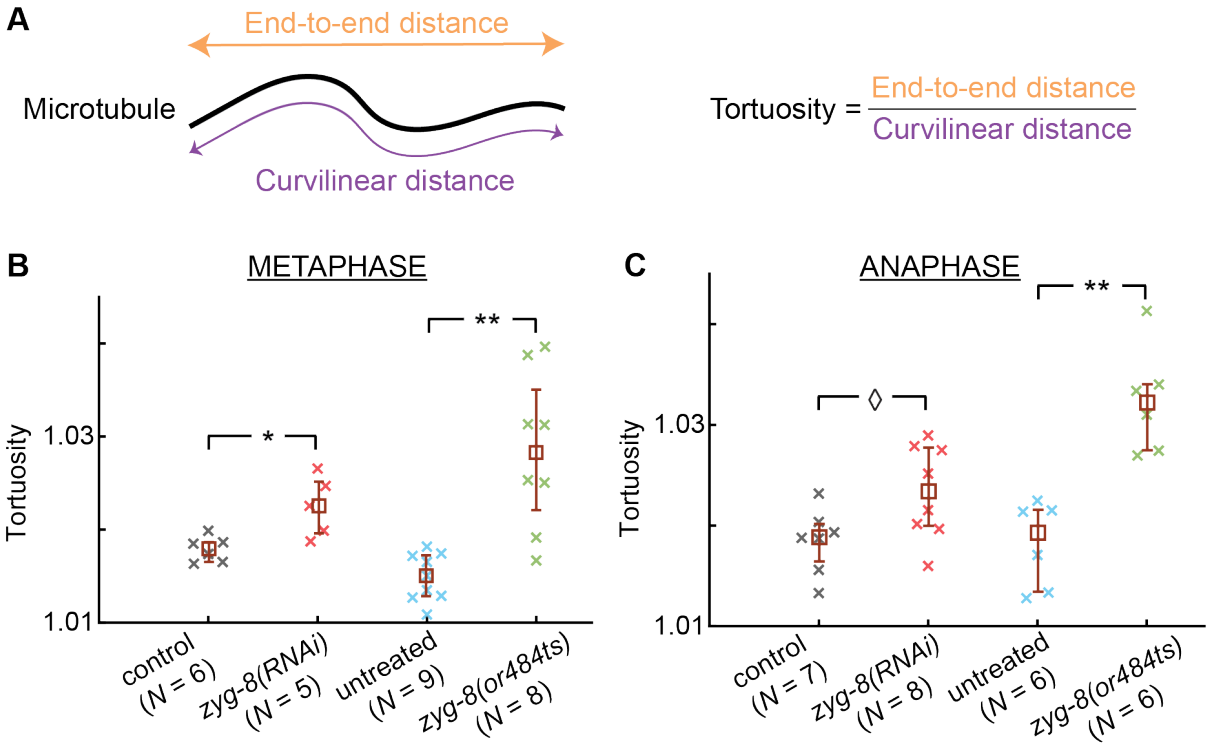

Figure 3: Astral microtubule tortuosity increases upon targeting *zyg-8*.
(A) Schematic about microtubule tortuosity measured as the ratio of curvilinear-distance over end-to-end distance. (**B-C**) Medians per z-projected stacks of microtubule tortuosities: (B) during metaphase using the same embryos as for Figure 2A, C, E and (C) during anaphase using the same embryos as for Figure 2B, D, F (Method M7). The brown squares and error bars correspond to the medians and the quartiles (Method M14).

2- ZYG-8 controls the cortical contact durations of astral microtubules through their flexural rigidity.

We, along with other researchers, have recently suggested that microtubule pushing against the cell cortex contributes to centring the spindle in metaphase and that microtubule buckling increases the stability of that process (Bouvrais et al., 2021; Garzon-Coral et al., 2016; Pécréaux et al., 2016). We reckoned that decreasing microtubule flexural rigidity would diminish the pushing exerted by each buckled microtubule (Euler force, buckling instability at critical compression force), consequently increasing its lifetime at the cortex (Brun et al., 2009; Janson et al., 2003). We first used the established agent-centred simulation *Cytosim* to test this hypothesis by varying microtubule rigidity (Figure S5A, Table S2, Movies S1-S3, Method M8) (Nedelec and Foethke, 2007). Similar to what we observed upon *zyg-8(RNAi)* and in *zyg-8(or484ts)* mutants, we found that both the highest microtubule curvatures (95th percentile) and the microtubule tortuosity increased when microtubule rigidity decreased (Figure S5B-C). Importantly, we measured that cortical lifetimes were increased when microtubule rigidity was reduced (Figure S5D). Thus, *in silico* analysis validated our hypothesis and showed that varying microtubule rigidity was enough to affect oppositely astral microtubule cortical lifetimes.

We next measured the cortical contact durations of the astral microtubules *in vivo* (Bouvrais et al., 2021; Bouvrais et al., 2018) (Method M9). We found a significant increase in the contact durations upon *zyg-8(RNAi)* during metaphase (Figure 4A-C). Conversely, *zyg-8* overexpression led to slightly shorter contact durations than untreated embryos (Figure 4D-F). These duration variations were consistent with ZYG-8 rigidifying the microtubules. Overall, we found that targeting *zyg-8* affected the microtubule rigidity, leading to cortical lifetimes of astral microtubules that varied inversely with their rigidity.

Our previous work demonstrated that cortical lifetime variations could also result from perturbing microtubule dynamics (Bouvrais et al., 2021). As the Doublecortin family may regulate microtubule growth in certain conditions (Bellanger et al., 2007; Dema et al., 2024; Lin et al., 2000; Srayko et al., 2005), we assessed whether the astral microtubule growth rate was altered upon *zyg-8* targeting using an EB-labelling of microtubule plus-ends (Method M10). In *zyg-8(or484ts)* mutants at the restrictive temperature, we observed a significant reduction of 16% (Figure S6A), similar to the previous *zyg-8(RNAi)* depletion through injection (Srayko et al., 2005). However, we found no significant change upon *zyg-8(RNAi)* or *zyg-8* overexpression (Figure S6B-C). The latter observations suggested that increased or decreased cortical lifetimes upon *zyg-8(RNAi)* treatment or *zyg-8* overexpression cannot be explained by a significant variation in the microtubule growth rate.

Longer durations of cortical microtubule contacts could also originate from more astral microtubules reaching the cortex, thus reducing the force born by each of them. Besides, a slight nucleation rate increase has been previously observed following *zyg-8(RNAi)* treatment through injection (Srayko et al., 2005). We thus counted the comet-like structures representing the EBP-2::mKate2 labelled plus-ends of growing microtubules emanating from the centrosomes to investigate the nucleation rate (Method M10). We measured a mild increase in the nucleation rate with *zyg-8(or484ts)* mutants and a mild decrease upon *zyg-8* overexpression, while no significant change was found upon *zyg-8(RNAi)* (Figure S7A-C). As the diameter of the centrosome is associated with its nucleation power (Baumgart et al., 2019; Decker et al., 2011; Piehl et al., 2004), we also measured the diameters of fluorescently labelled centrosomes using mCherry::TBG-1 (Method M12). We found a significant decrease in diameter upon *zyg-8* overexpression (Figure S7D). We also observed a slight increase in

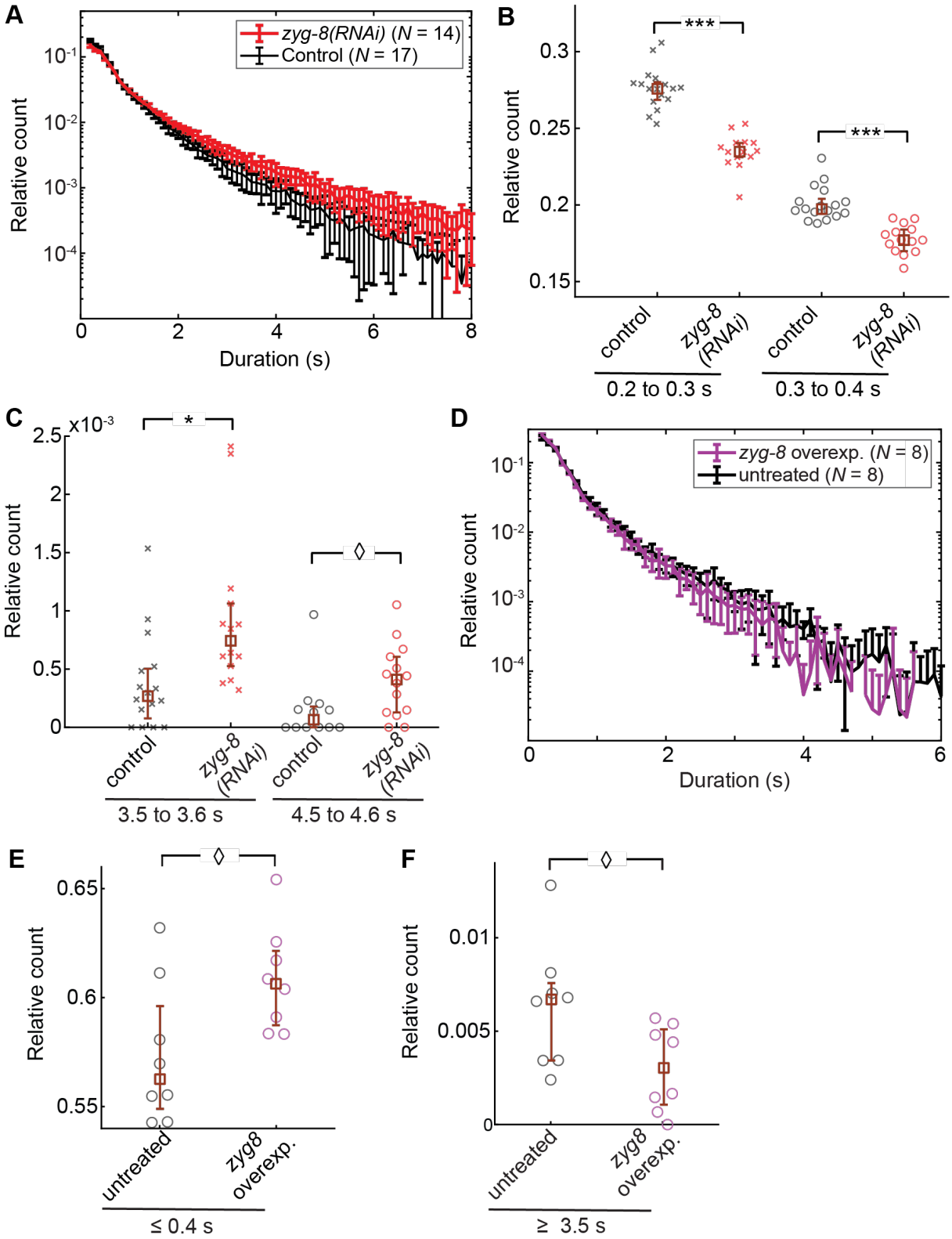

Figure 4: Cortical lifetimes of astral microtubules in metaphase anticorrelate with ZYG-8 expression level.

(**A**) Embryo-averaged and normalised distributions of the cortical lifetimes of astral microtubules during metaphase. We tracked and analysed the cortical contacts of microtubules labelled by GFP::TBB-2 in (red) N =14 *zyg-8(RNAi)* treated embryos and (black) N = 17 control ones (Kolmogorov–Smirnov test: $p = 6\text{x}10^{-4}$, Methods M9 and M14). (**B-C**) Relative counts for (B) the two lowest duration

bins (0.2 to 0.3 s & 0.3 to 0.4 s) and (C) two longer duration bins (3.5 to 3.6 s & 4.5 to 4.6 s) of the distributions presented in (A) to highlight variations in the relative counts of the shortest durations or the longest ones. (**D**) Embryo-averaged and normalised distributions of the cortical lifetimes of astral microtubules during metaphase. We tracked and analysed the cortical contacts of microtubules labelled by mCherry::tubulin in (purple) N = 8 *zyg-8* overexpressing embryos and (black) N = 8 untreated ones (Kolmogorov–Smirnov test: p = 0.18, Methods M9 and M14). (**E-F**) Relative counts for (E) the durations below 0.4 s and (F) the durations above 3.5 s of the distributions presented in (D) to highlight variations in the relative counts of the shortest and longest durations. The brown squares and error bars correspond to the medians and the quartiles (Method M14).

diameter following *zyg-8(RNAi)* treatment (Figure S7E). Comparatively, a mild depletion of SPD-2^{CEP192} led to a much larger reduction in centrosome diameter (Figure S7F) (Woodruff et al., 2015). Overall, our measurements confirmed that ZYG-8 limits the microtubule nucleation rate, although to a lower degree. Consequently, we proposed that the variations in microtubule cortical contact durations upon targeting *zyg-8* cannot be explained by the sole mild perturbation of the microtubule nucleation rate. Accounting for the perturbation of microtubule flexural rigidity enabled explaining the changes in microtubule cortical contact durations upon targeting *zyg-8*.

From a functional standpoint, ZYG-8 regulates the flexural rigidity of astral microtubules, influencing their cortical residence times and likely playing a key role in proper spindle positioning.

3- **ZYG-8 regulates the centring force through microtubule rigidity.**

We foresaw that we could target *zyg-8* to reveal the contribution(s) of microtubule rigidity in spindle positioning during anaphase. Indeed, centring force through microtubules pushing against the cell cortex, mostly invoked in metaphase, depends essentially on the microtubule rigidity (Bouvrais et al., 2021; Garzon-Coral et al., 2016; Pécréaux et al., 2016). This force is also expected to serve as a restoring force during the rocking of the spindle in anaphase (Grill et al., 2005; Pécréaux et al., 2006a). Upon *zyg-8(RNAi)*, we observed a large increase in the spindle-pole oscillation amplitudes (Figure 5A, Method M11). Interestingly, we also noticed a significant reduction in the frequency of these oscillations, consistent with softer microtubules (Figure 5B) (Howard, 2006; Pécréaux et al., 2006a). We considered two potential and non-exclusive mechanisms for the increased oscillation amplitudes: enhanced cortical pulling or reduced cortical pushing. First, we measured the most-posterior position of the posterior centrosome during anaphase following *zyg-8(RNAi)* treatment and found no significant difference. It suggested that the posterior displacement under the control of cortical pulling force was substantially unaltered (Figure S8). Next, we applied the DiLiPop analysis to assess cortical pulling and pushing events during anaphase, based on a statistical analysis of the cortical contact durations of astral microtubules (Bouvrais et al., 2021) (Method M9). For the pulling events, we observed a modest rise in their lifetime by 10% and a slight reduction in their density by 3% (Figure 5C). Since all cortical force generators are engaged in pulling events during anaphase, the reduced density following *zyg-8(RNAi)* treatment likely reflected the longer duration of each pulling event (Bouvrais et al., 2018; Grill and Hyman, 2005). We concluded that *zyg-8(RNAi)* treatment did not significantly affect the cortical pulling force. In contrast, we found that pushing microtubules resided at the cortex for a longer duration (+37%) upon *zyg-8(RNAi)*, which aligned with their dependence on microtubule rigidity (Figure 5D1). Besides, we observed a significant increase in the density of pushing events by

22%, likely due to the nucleation rate increase (Figure 5D2). We proposed that the larger oscillation amplitudes seen following *zyg-8(RNAi)* treatment resulted from reduced cortical pushing force, despite the rise in pushing event density. We attributed this force reduction to softer microtubules, leading to less efficient pushing events.

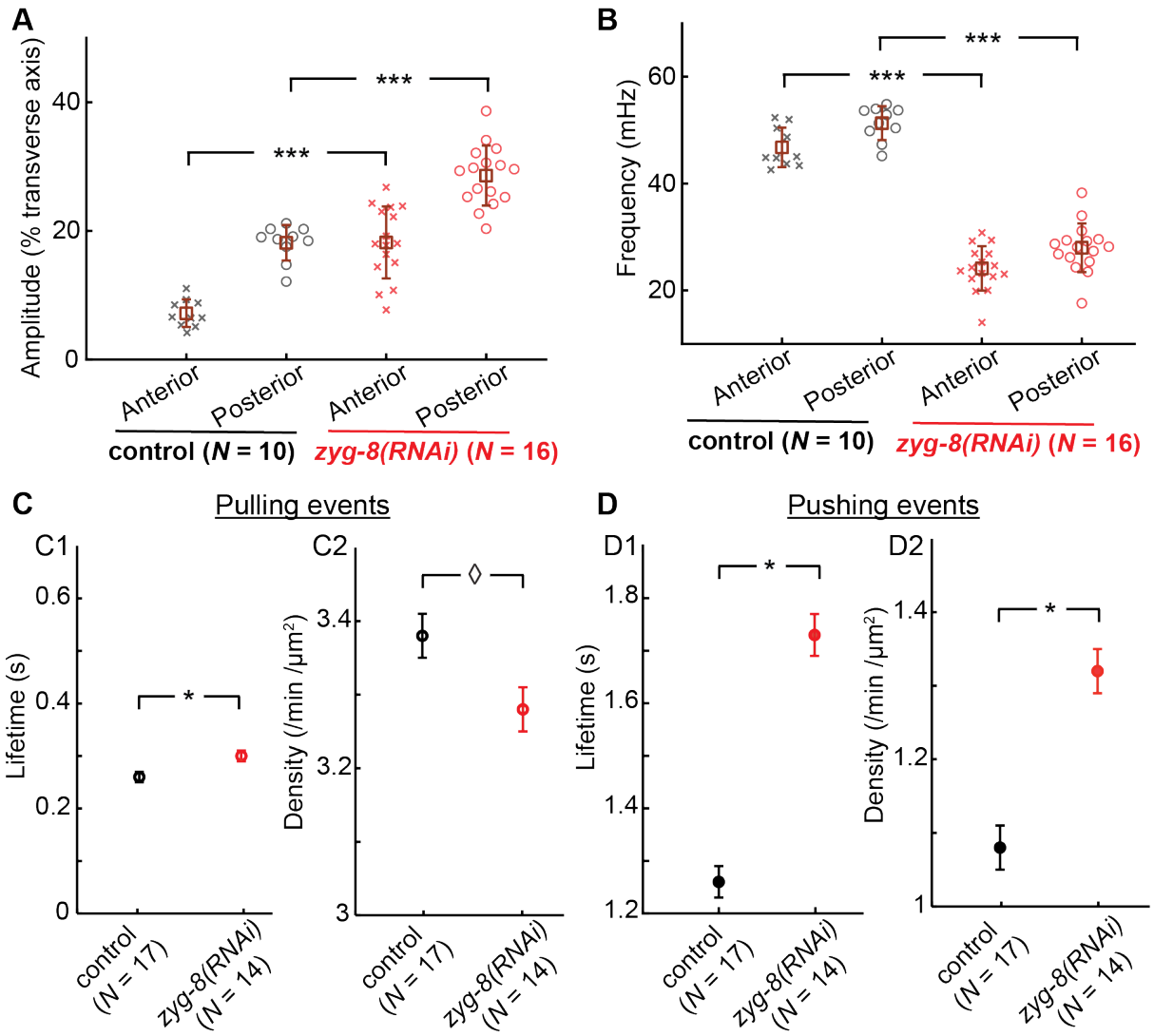

Figure 5: Depleting ZYG-8 increases the spindle pole oscillation amplitudes and perturbs highly the cortical pushing events.

(**A**) Maximal peak-to-peak amplitudes of spindle pole oscillations and (**B**) frequencies of the oscillations. We tracked and analysed the centrosomes using a GFP::TBB-2 fluorescent labelling in (red) *N* = 16 *zyg-8(RNAi)* treated embryos and (black) *N* = 10 control embryos (Method M11). The brown squares and error bars correspond to the means and SD (Method M14). (**C**) Lifetimes and densities of the cortical pulling events, and (**D**) lifetimes and densities of the cortical pushing events. We tracked and analysed the microtubule contacts at the cell cortex using a GFP::TBB-2 fluorescent labelling in (red) *N* = 14 *zyg-8(RNAi)* treated embryos and (black) *N* = 17 control embryos (same embryos as for Figure 4A-C) (Methods M9 & M14).

We sought an alternative characterisation of spindle positioning forces at the cellular level to ascertain ZYG-8 role in changing microtubule rigidity and centring force generated by microtubules pushing against the cortex. To do this, we extracted the mechanical fingerprint of spindle positional micro-movements along the transverse axis during anaphase and fitted

the experimental spectrum to a second-order model (Pécréaux et al., 2016) (Method M13). Our results showed that ZYG-8 depletion led to a large reduction in the centring-to-damping corner frequency f_c, which decreased by 79% compared to the control (Table S3). We also observed a decrease in the diffusion coefficient D (scaling inversely to damping) by 24% and an increase in the damping-to-inertia frequency f_0, which rose by 35% (Table S3). We interpreted the decrease in f_c as primarily reflecting a centring force reduction (Pécréaux et al., 2016). The modest change in D was likely due to more microtubules contacting the cortex. f_0 increase would be partly due to the reduced D, and appeared hardly compatible with any significant increase in inertia. It suggested that the pulling forces were only mildly or not affected. Overall, the spindle positional mechanical fingerprint following *zyg-8(RNAi)* treatment was qualitatively consistent with a reduction in the centring force, likely due to softer microtubules.

Next, we investigated whether *zyg-8* overexpression would affect spindle pole oscillation. We observed no significant variation in the maximal amplitudes, but there was a significant increase in oscillation frequency, consistent with stiffer astral microtubules (Figure S9A-B). Additionally, we found no change in the most-posterior position of the posterior centrosome (Figure S9C). Using the DiLiPop assay, we revealed that the lifetime of pulling events was not significantly affected, and their density was slightly reduced (Figure S9D). Both sets of experiments suggested that the cortical pulling force was not significantly altered by *zyg-8* overexpression. In terms of pushing events, DiLiPop analysis revealed a slight reduction in their lifetime with no change in their density (Figure S9E). We also studied spindle positional micro-movements along the transverse axis in embryos overexpressing *zyg-8* during anaphase. Fitting the experimental spectrum of these micro-movements suggested no major change in centring-to-damping corner frequency, indicating that centring efficiency was unaffected by *zyg-8* overexpression (Table S3). Altogether, the DiLiPop analysis and the positional micro-movements study indicated no substantial variation in the cortical pushing/centring forces upon *zyg-8* overexpression, consistent with the unaffected spindle pole oscillation amplitudes. Therefore, we proposed that the increase in microtubule rigidity upon *zyg-8* overexpression was not large enough to have a functional impact.

Given that we observed a modest regulation of microtubule nucleation by ZYG-8, we questioned to what extent this could contribute to the phenotypes described above. To do so, we set out to compare the mechanical fingerprint of spindle positional micro-movements during anaphase upon *zyg-8* overexpression and SPD-2^{CEP192} depletion, as both treatments are known to reduce the nucleation rate (Srayko et al., 2005). We performed a mild *spd-2(RNAi)*, leading to a more significant reduction in centrosome diameter than *zyg-8* overexpression (Figure S7D, F). When we fitted the experimental spectrum of the spindle transverse positional micro-movements in the *spd-2(RNAi)* condition, we found an increase in D and f_c, in contrast to the effects of *zyg-8* overexpression (Table S3). Based on these results, we proposed that the differences observed in the mechanical parameters upon *zyg-8* overexpressing were likely driven by an increase in microtubule rigidity, rather than by changes in the nucleation rate. Similarly, we suggested that the variation of this rate only modestly contributed to *zyg-8(RNAi)* mechanical fingerprint phenotype.

Altogether, our results showed that ZYG-8 regulated the cortical pushing force, causing centring, primarily by controlling microtubule flexural rigidity. This force acted as a restoring force during anaphase spindle rocking. Our work highlighted that this force contributed to

spindle choreography, although cortical pulling forces that drive the spindle posterior displacement remain dominant.

4- Very soft microtubules cause strong defects in spindle final positioning and orientation.

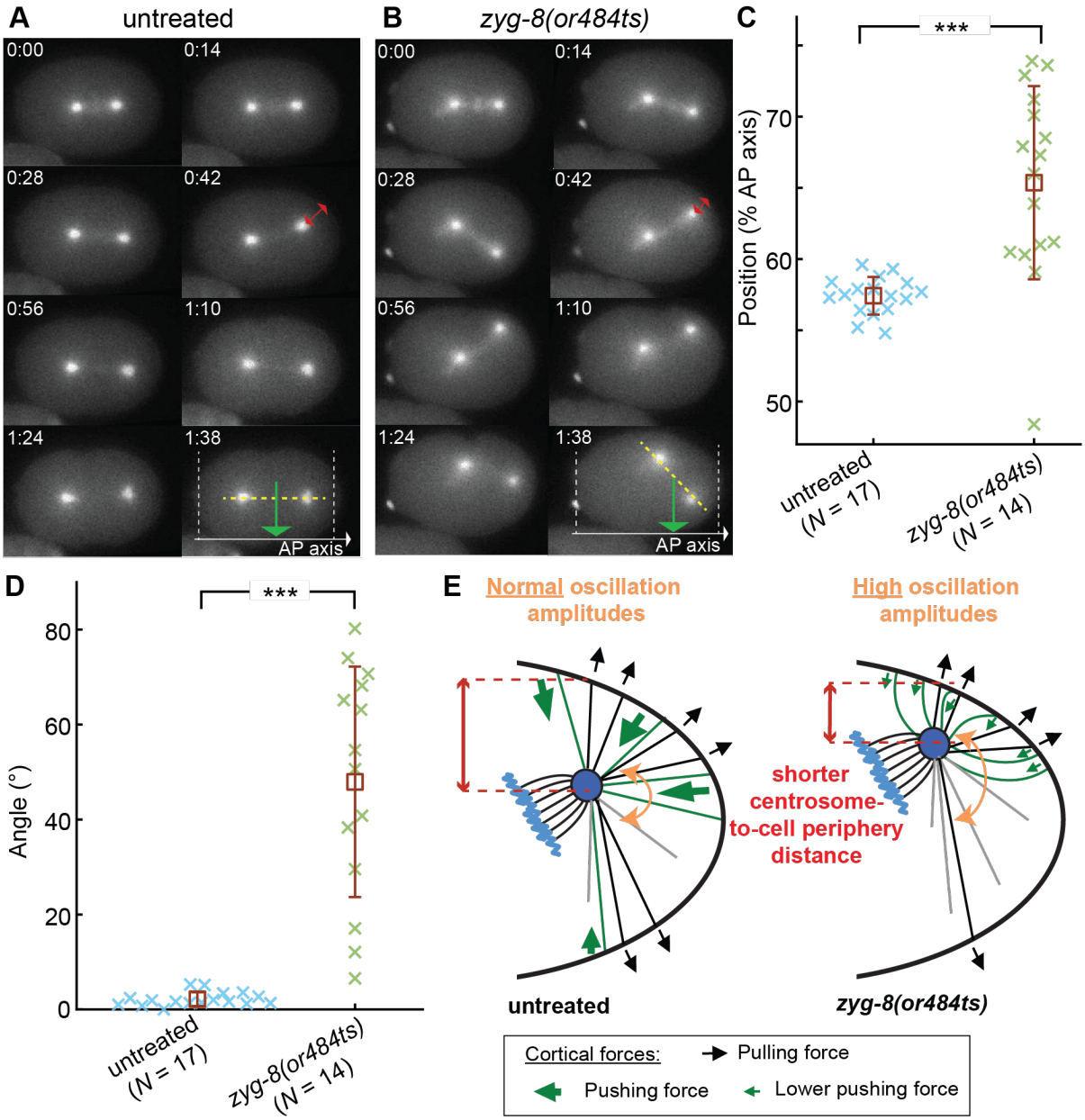

Figure 6: In *zyg-8(or484ts)* mutants, overly soft microtubules result in a misoriented and mispositioned spindle at the anaphase end.

(**A-B**) Time-lapse series of (A) an exemplar untreated embryo and (B) an exemplar *zyg-8(or484ts)* mutant along anaphase, both at the restrictive temperature and with EBP-2::mKate2 labelling. The green arrows indicate the spindle's final positions along the anteroposterior (AP) axis and the dotted yellow lines indicate the final orientations. The red double-headed arrows underline the minimal centrosome-to-cortex distances. The scale bars represent 10 µm. (**C-D**) Spindle final (C) positions along the AP axis and (D) angles for (light green) *N* = 14 *zyg-8(or484ts)* mutants and (light blue) *N* = 17 untreated embryos, both at the restrictive temperature. We tracked and analysed the centrosomes of embryos with EBP-2::mKate2 labelling (Method M11). The brown squares and error bars correspond

to the means and SD (Method M14). (**E**) Schematics illustrating how a large reduction in microtubule flexural rigidity could lead to spindle positioning and orientation defects.

After revealing a novel role for ZYG-8 in controlling microtubule flexural rigidity, we investigated its relevance to mitosis success. Interestingly, in *zyg-8(or484ts)* mutants at the restrictive temperature, we observed larger spindle-pole oscillation amplitudes compared to the *zyg-8(RNAi)* treatment (Figure S10A). We attributed this phenotype to weakened centring caused by overly soft microtubules, which was supported by a larger and significant reduction in the centring-to-damping corner frequency f_c and increased proportion and magnitude of microtubule curvatures (Table S3, Figures 2 and 3). It resulted in a significantly reduced centrosome-to-cortex distance. We thus explored the consequences of this reduced distance in the course of anaphase. In untreated embryos, oscillations progressively build up and then die down, leaving the spindle aligned along the embryo anteroposterior (AP) axis (Figure 6A). However, in *zyg-8(or484ts)* mutants at the restrictive temperature, the centrosome's proximity to the cell periphery pulled the entire spindle further toward the cell cortex and seemed to prevent the spindle from returning to the centreline during the oscillation die-down (Figure 6B). Reduced pushing disrupted the force balance, favouring excessive pulling. As a result, the spindle could not return to the central position at the anaphase end, leading to mispositioning and misorientation (Figure 6B-D). Based on these observations, we proposed that the misoriented spindle at the anaphase end in *zyg-8(or484ts)* mutant was due to impaired pushing forces.

A previous and alternative explanation suggested that these defects in the mutants could be due to very short astral microtubules that failed to reach the opposite cortex (Bellanger et al., 2007; Gönczy et al., 2001). This was supported by observations of very short astral microtubules in fixed *zyg-8(or484ts)* mutant stained with anti-tubulin at the anaphase stage. However, in our experiments, we observed this prominent phenotype in only a small fraction of fixed mutants (33%). To safeguard against possible artefacts, especially given that the reduced microtubule rigidity might make them more fragile to fixing, we imaged live embryos with a GFP::TBB-2 fluorescent labelling. In *zyg-8(or484ts)* mutants at the restrictive temperature, we observed exaggerated rocking during anaphase. At the end of mitosis, the spindle was positioned more posteriorly and tilted (Movies S4, S5 compared to Movie S6 for control). Interestingly, the astral microtubules were long enough to reach the cell periphery, even during anaphase spindle-pole oscillations (Figure S10B, Movies S4, S5). Additionally, we observed a larger number of highly curved astral microtubules in the mutants, confirming our curvature and tortuosity measurements in fixed embryos (Figures 1-3). Based on these observations, we proposed that the positional defects observed in *zyg-8(or484ts)* mutants at the restrictive temperature were caused by overly soft microtubules rather than microtubules that were too short.

In conclusion, sufficiently rigid microtubules were essential to generate enough centring force in anaphase to prevent the centrosome from being displaced too far from the centre and ensure proper final orientation of the spindle (Figure 6E). This reproducible spindle displacement guaranteed the proper positioning of the spindle at the mitosis end. As the latter dictates the furrow ingression position, it also ensures the correct partitioning of cell fate determinants.

5- PTL-1, like ZYG-8, reduced the centring forces, although to a lower extent.

Since ZYG-8 has been involved in other functions during cell division, we set out to target another protein known to regulate microtubule flexural rigidity in neurons and test whether we could reproduce similar perturbations in spindle choreography. We depleted the sole nematode homolog of the Tau family, PTL-1, using RNAi (Goedert et al., 1996; McDermott et al., 1996). We measured a modest increase in the maximal amplitudes of spindle pole oscillations and a reduction in their frequencies, consistent with softer microtubules (Figure 7). Besides, when examining the spindle positional micro-movements along the transverse axis during anaphase, we found that the centring-to-damping corner frequency f_c was reduced (Table S3). These results closely resembled those obtained with *zyg-8(RNAi),* suggesting a reduction in centring forces, albeit to a lesser degree. These phenotypes were comparatively weaker likely since the level of *ptl-1* expression in the zygote was lower compared to *zyg-8* (Tintori et al., 2016). Based on these findings, we proposed that PTL-1 also controlled microtubule flexural rigidity and contributed to spindle choreography during *C. elegans* zygote division. Furthermore, our results with PTL-1 reinforced the crucial role of ZYG-8 in controlling microtubule flexural rigidity to ensure proper spindle positioning and orientation.

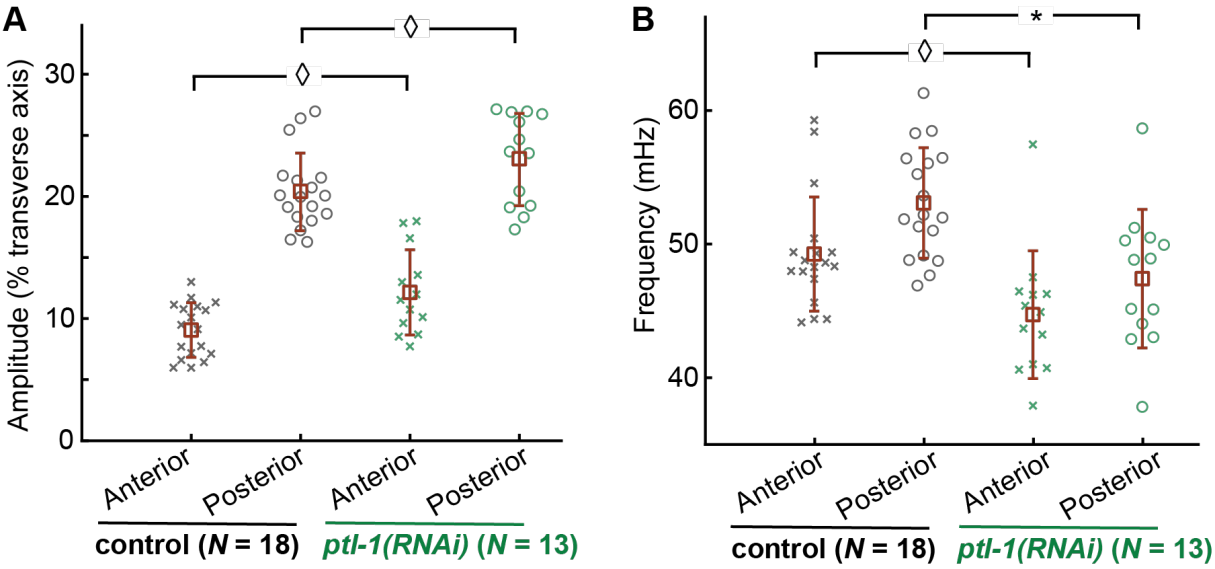

Figure 7: *ptl-1(RNAi)* leads to larger spindle-pole oscillation amplitudes during anaphase, similar to *zyg-8(RNAi)*, although to a lower extent.

(**A**) Maximal amplitudes of spindle pole oscillations and (**B**) frequencies of the oscillations for (green) *N* = 13 *ptl-1(RNAi)* treated embryos and (black) *N* = 18 control embryos. We tracked and analysed the centrosomes labelled by mCherry::TBG-1 (Method M11). The brown squares and error bars correspond to the means and SD (Method M14).

DISCUSSION

Our work proposes that ZYG-8 is a major player in *C. elegans* mitosis, mainly through rigidifying the microtubules. This contribution is crucial for generating cortical pushing forces large enough to counteract the intense cortical pulling forces during anaphase. Proper force balance during anaphase ensures accurate spindle orientation at the end of mitosis.

Previous research on mitotic spindle positioning in the *C. elegans* zygote has highlighted the crucial roles of microtubule dynamics and asymmetric cortical pulling forces controlled by PAR polarity (Bouvrais et al., 2021; Bouvrais et al., 2018; Fielmich et al., 2018; Grill et al., 2001; Grill et al., 2003; Kozlowski et al., 2007; Nguyen-Ngoc et al., 2007; Pécréaux et al., 2016;

Redemann et al., 2010; Wu and Rose, 2007). Besides, cortical pushing forces have been proposed as centring forces during metaphase (Garzon-Coral et al., 2016; Pécréaux et al., 2016). The current study complements and departs from these previous investigations**.** Firstly, it reveals that controlling microtubule flexural rigidity participates in the spindle choreography. Particularly, this control avoids excessive closeness of the spindle poles to the cell periphery during anaphase. We indeed observed increased spindle pole oscillation amplitudes following ZYG-8 or PTL-1 depletion (Figures 5A & 7A). This control through microtubule rigidity is complementary to the well-known regulation through microtubule dynamics. Secondly, our study underlines that cortical pushing force also contributes to spindle positioning during anaphase, although cortical pulling forces are dominant and their asymmetry ensures spindle posterior displacement (Grill et al., 2003; Nguyen-Ngoc et al., 2007). It is often assumed that the cortical pushing force plays little to no role due to the comparatively high intensity of cortical pulling forces, especially since microtubule buckling reduces cortical pushing forces (Dogterom et al., 2005; Farhadifar et al., 2020; Wu et al., 2023). We propose that weakening the cortical pushing forces, as observed in *zyg-8(or484ts)* mutants, hinders the spindle's aligning along the AP axis (Figure 6B). This disruption precludes the correct final position and orientation of the spindle, ultimately affecting the accurate segregation of fate-determining factors (Figure 6C-D). Here, we show that astral microtubules in *zyg-8(or484ts)* mutants are capable of contacting the opposite cortex (Figure S10B, Movies S4 and S5), which differed from Kozlowski work suggesting that limited access of astral microtubules to the cell cortex caused spindle oscillation (Kozlowski et al., 2007). This observation rules out a mis-orientation grounded on limited cortical access by the microtubules. Overall, the astral microtubule network functions like a spring, restricting the spindle transverse motion away from the centre in *C. elegans* zygotes (Garzon-Coral et al., 2016; Howard, 2006; Pécréaux et al., 2016). Therefore, microtubule flexural rigidity emerges as a critical physical parameter in controlling the centring force intensity.

We foresee that the essential role for pushing goes beyond the nematode's first division and may be relevant to other asymmetric divisions and maybe further. It is well established that the pulling dominates the spindle orientation. It is accounted for by the canonical model based on the molecular machinery composed of the cortical $G\alpha$-LGN^{GPR}-$NuMA^{LIN-5}$ complex and the cytoplasmic dynein motor (Bergstralh et al., 2017; di Pietro et al., 2016). However, less attention has been paid to the contribution of the pushing forces to spindle orientation. The dependence of spindle orientation on cell shape, which is often neglected, suggests that the pushing force could play a significant role in this process (Jingchen et al., 2019; Mao et al., 2011; Singh et al., 2021; Strauss et al., 2006; Tsou et al., 2002). In other systems, some studies found that the spindle did not align with the dynein-enriched cortical zones (Izumi et al., 2006; Saleh et al., 2023; Siller et al., 2006; Williams et al., 2011). Consequently, the traditional canonical model alone cannot account for spindle orientation in various contexts. We propose that the cortical pushing force contributes to this phenomenon and that the flexural rigidity of microtubules must be sufficiently high. Notably, the orientation of the mitotic spindle defines the direction in which a cell divides. Therefore, many developmental processes, ranging from germline stem cell division to epithelial tissue homeostasis and regeneration, require correct mitotic spindle orientation. Deficits in spindle orientation have been associated with various diseases, such as organogenesis and morphogenesis failures, or cancer (Baena-López et al., 2005; Fededa et al., 2016; Fischer et al., 2006; Fleming et al., 2009; Gao et al., 2017; Pease and Tirnauer, 2011; Quesada-Hernández et al., 2010; Quyn et al., 2010).

Our study revealed that ZYG-8^{DCLK1} plays a crucial role in regulating the flexural rigidity of microtubules during zygote division, marking a novel function for this protein in mitosis. Looking ahead, it will be intriguing to explore the mechanisms through which ZYG-8 rigidifies the microtubules. Several mechanisms have been proposed, primarily in the context of neurons. We take advantage of the numerous studies on the DCX protein, which shares the two same microtubule-binding domains as DCLK1^{ZYG-8}. Besides, these two doublecortin domains are highly conserved in ZYG-8 (Gönczy et al., 2001). (1) Microtubules can be rigidified by hindering the sliding of protofilaments through the binding of MAPs across these protofilaments. DCX binding on the microtubules is compatible with this mechanism since it reinforces lateral and longitudinal dimer couplings. (2) Rigidification can also occur by bundling multiple microtubules together. Previous studies revealed such a mechanism for the Doublecortin family *in vitro* and *in vivo* (Bellanger et al., 2012; Bielas et al., 2007; Couillard-Despres et al., 2004; Horesh et al., 1999; Kim et al., 2003; Lin et al., 2000; Moores et al., 2006). In particular, *zyg-8(or484ts)* mutants displayed a perturbed bundle organisation of microtubules in touch receptor neurons (Bellanger et al., 2012). However, in the *C. elegans* zygote, electron microscopy failed to reveal microtubule bundles in the spindle or astral microtubules close to the poles (Redemann et al., 2017). Therefore, we disfavoured such a mechanism. (3) Microtubule rigidity is also regulated through post-translational modifications (PTM) (Nishi et al., 2023; Portran et al., 2017; Robison et al., 2016; Wall et al., 2020; Xu et al., 2017). Notably, a study showed that DCX expression increases tubulin polyglutamylation in neurons (Sébastien et al., 2024). However, the *ttll-4(-);ttll-5(-);ttll-11(-)* triple mutants, which prevented tubulin glutamylation, did not show any defect in embryonic viability. It suggested that microtubule glutamylation may not significantly affect the zygote cell division (Chawla et al., 2016). (4) It was previously shown that damages along microtubule lattice soften microtubules (Aher et al., 2020; Schaedel et al., 2015). Thus, self-repair of local damages may also help restore the rigidity or stabilise the curved region (Théry and Blanchoin, 2021). Interestingly, the number of microtubule defects could notably correlate with the microtubule growth rate (Chrétien et al., 1992; Schaedel et al., 2019). The latter is particularly high in nematodes (Chaaban et al., 2018). If a high occurrence of holes exists in the *C. elegans* embryo, ZYG-8 could contribute to healing as the Doublecortin family also acts as tubulin oligomer chaperones (Manka and Moores, 2020). However, to date, there has been no evidence of microtubule lattice defects in *C. elegans* zygote. For example, embryos with EBP-2 fluorescent labelling did not show stationary fluorescent spots along the astral microtubules (Kozlowski et al., 2007; Srayko et al., 2005). The various mechanisms proposed above, in which ZYG-8 could play a key role in regulating microtubule flexural rigidity, are not mutually exclusive; several of them may be combined. ZYG-8 rigidifying the microtubule by coupling the protofilaments appears to us the most plausible mechanism currently.

From a broader perspective, we propose a dual role of ZYG-8^{DCLK1} during mitosis, acting by regulating both microtubule rigidity and dynamics. Our research has corroborated Srayko's findings that ZYG-8 enhances microtubule growth while limiting the nucleation of astral microtubules in *C. elegans* zygote (Figures S6 and S7) (Srayko et al., 2005). The present study emphasises that the spindle choreography phenotypes upon *zyg-8(RNAi)* were mainly caused by the disturbed microtubule rigidity, rather than the modest changes in microtubule dynamics. Why ZYG-8 plays a greater role in regulating microtubule rigidity rather than microtubule dynamics. This is likely because ZYG-8 and EB compete for the same microtubule binding site at the corner of four tubulin dimers (Fourniol et al., 2010; Manka and Moores, 2018; Maurer et al., 2012). However, EB binds preferentially to the GTP cap at growing ends

(Maurer et al., 2012), while Doublecortin-family proteins bind *in vitro* to the whole lattice (Bechstedt and Brouhard, 2012). *In vivo*, DCX was excluded from microtubule ends (Ettinger et al., 2016). A relative exclusion of ZYG-8 from microtubule ends in *C. elegans* zygote could account for the efficiency differences in its dual role. This dual role may be particularly relevant in specific cellular contexts (e.g., neurons) where proteins essential for microtubule growth or nucleation (e.g., γ-tubulin ring complexes) are lacking or in low abundance (Nguyen et al., 2011; Stiess et al., 2010). Interestingly, proteins from the Doublecortin family are highly expressed in neurons, where they regulate microtubule rigidity (Bellanger et al., 2012; Jean et al., 2012). They may also contribute there to microtubule nucleation and/or growth, consistent with their *in vitro* capabilities (Lin et al., 2000; Manka and Moores, 2020; Moores et al., 2004; Moores et al., 2006). Collateral branching in hippocampal neurons was significantly delayed following DCX depletion, which may reflect nucleation impairment (Tint et al., 2009). Overall, ZYG-8^{DCLK1} performs multiple microtubule-related functions in a context-dependent manner; we suggest controlling flexural rigidity as a novel one during cell division and possibly beyond.

By uncovering ZYG-8's dual role in *C. elegans* mitosis, we can now question its contribution to zygote meiosis, especially through controlling microtubule flexural rigidity. A recent study highlighted that ZYG-8 promotes meiotic spindle stability and suggested that it may regulate microtubule dynamics and motor-driven forces (Czajkowski et al., 2024). We wonder whether some of the perturbations that they observed upon targeting *zyg-8* could be attributed to the deregulation of microtubule rigidity. Notably, they found that most *zyg-8(or484ts)* meiotic spindles were significantly bent at the restrictive temperature. Additionally, some images of the mutant showed microtubules exhibiting high local curvatures. Similar perturbations were also observed following ZYG-8 depletion using the Auxin-inducible degradation system. In light of these observations, it is plausible that ZYG-8 contributes to the assembly or/and stability of meiotic spindles in the zygote by stiffening microtubules.

In conclusion, our findings regarding the pivotal role of ZYG-8^{DCLK1} in spindle positioning and orientation through rigidifying microtubules during mitosis offer not only a deeper understanding of the fundamental mechanisms governing cell division but also highlight significant implications for cancer therapy. Given that DCLK1^{ZYG-8} is frequently deregulated in various solid tumours (e.g., those from the colon, pancreas, kidney and breast), this suggests its involvement in a common process such as cell division (Gao et al., 2016; Ge et al., 2018; Ito et al., 2016; Liu et al., 2019). Additionally, as the proteins of the Tau family are also found to be deregulated or mutated in cancers, targeting microtubule rigidity may represent a promising approach for future cancer treatments (Gargini et al., 2019; Papin and Paganetti, 2020; Rossi et al., 2018). This strategy could potentialise existing therapies targeting microtubule dynamics, which often suffer from side effects and are limited by cancer cell resistance.

MATERIAL AND METHODS

M1- C. elegans strains and their culturing

The *C. elegans* strains used in the present study are listed in Table S1. *C. elegans* nematodes were cultured as described in (Brenner, 1974) and dissected to obtain embryos. The strains were maintained at 20°C, except the thermosensitive mutant *zyg-8(or484ts)* maintained at 15°C and AZ244 maintained at 25°C to increase transgene expression. The strains were handled on nematode growth medium (NGM) plates and fed with OP50 *E. coli* bacteria.

M2- RNAi feeding

RNA interference (RNAi) experiments were performed by feeding, as described in (Kamath and Ahringer, 2003). Bacteria were obtained by Source BioScience (*zyg-8*: III-6C10; *ptl-1*: III-1A12; *spd-2*: I-4O08). The feedings were performed at 20°C. For *zyg-8* and *ptl-1* RNAi, the treatment lasted 96h and dsRNA expression was induced with 4 mM of IPTG (Isopropyl β-D-1-thiogalactopyranoside). For *spd-2* targeting, the duration was 6h, and dsRNA expression was induced with 1mM IPTG. The control embryos for the RNAi experiments were fed with bacteria carrying the empty plasmid L4440.

M3- Preparation of the embryos for live imaging or immunofluorescence

For live imaging, embryos were dissected in M9 buffer (prepared by combining 3 g KH_2PO_4, 6 g Na_2HPO4, 5 g NaCl and 1 mL of 1 M $MgSO_4$ diluted in ultrapure water to 1 L) and mounted on a pad (2% w/v agarose, 0.6% w/v NaCl, 4% w/v sucrose) between a slide and a coverslip.
For immunofluorescence staining, embryos were dissected in M9 buffer and mounted on a slide coated with poly-L-lysine (0.1%) (Sigma-Aldrich P1524). The slides were dipped in liquid nitrogen to crack the embryo's eggshell and immediately transferred to -20°C methanol for fixation for 20 minutes (freeze-cracking method (Duerr, 2013)). They were then rinsed for 10 minutes in PBS 1X before blocking for 20 minutes in PBS-Tween (0.2%) with BSA (1%).
Importantly, for the thermosensitive *zyg-8(or484ts)* mutants, we transferred them as L4 larvae to 25°C and incubated for 15 hours before use, as previously done in (Gönczy et al., 2001).

M4- Fluorescence microscopic imaging conditions

The various microscopy setups used are presented in the table below. Embryos were imaged at 23°C, except otherwise stated. Images were stored using the Omero software (Li et al., 2016).

Imaging experiment	**Spindle pole imaging**	**(1) Landing assay to study microtubule cortical contacts; (2) Microtubule dynamics (growth and nucleation rates)**	**(3) Microtubule network, (4) ZYG-8 localisation inside the embryo, (5) microtubule live imaging**
Live or fixed embryo	Live	Live	(3) & (4): Fixed; (5): Live
Microscope type	Zeiss Axio Imager upright	Leica DMi8 equipped with CSU-X1 spinning disk head (Yokogawa)	(3) & (4): Airyscan 2 Zeiss LSM980. (5) Airyscan Zeiss LSM880.
Fluorescent labelling	Spindle poles, e.g. mCherry::TBG-1	(1) Microtubules: GFP::TBB-2 or mCherry::tubulin.	(3) DM1A-AF488 conjugate.

		(2) Microtubule plus-ends : EBP-2::mKate2	(4) mNG::ZYG-8 and mCherry:tubulin. (5) GFP::TBB-2
Objective	100x/1.45 NA Oil plan-Apo	100x/1.4 NA Oil HCX Plan Apo	63x/1.4 NA Oil Apochromat DIC M27
Acquisition plane(s)	Spindle plane	(1) Cortex plane close to the glass slide (located 12-15 µm below the spindle plane). (2) For *zyg-8(RNAi)*: spindle plane; for *zyg-8* overexp. and *zyg-8(or484ts)*: ~3 µm below spindle plane	(3) 25-30 or (4) 10-12 z-sections centred at the spindle plane; z-step: (3) 0.17 µm, (4) 0.3 µm. (5) only the spindle plane.
Camera	Andor iXon3 EMCCD 512x512	ultra-sensitive Roper Evolve EMCCD	Airyscan detector
Illumination, excitation and emission characteristics	Illumination: mercury lamp. NG channel: excitation: enhanced transmission (ET) 485/12 bandpass (BP) (AHF analysentechnik); beamsplitter: low pass (LP) 495; emission: 525/50 ET Bandpass mCherry channel: excitation: BP 530-585; beamsplitter: 600; emission: low pass 615	Illumination: Argon laser mCherry channel: laser selecting the line at 561 nm with DAPI-GFP-mCherry-Cy5 emission filter and a Quad Dichroic filter. GFP channel: laser selecting the line at 488 nm with GFP/FITC 4 nm BP excitation filter and retrieving fluorescence with a Quad Dichroic emission filter	Illumination: laser diodes (3) excitation wavelength 488 nm; main beam splitter (MBS) 488/639 and 405. (4) excitation wavelength 488 nm or 561 nm; MBS 488/561 and 405; secondary beam splitter LP525 (for 488 nm laser) or SP550 (for 561 nm laser). (5) excitation wavelength 488 nm; MBS BP 420-480 + BP 495.
Spatial resolution (µm/pixel)	0.16	0.14	(3) & (4): 0.04. (5): 0.09
Temporal resolution	32.498 frame/s	(1) 10 frame/s (2) 2.5 frame/s	(3) 41.6 s per frame (4) 1 min 23 s per frame (5) 944 ms per frame
Additional parameters or equipment	Extra anti-heat and ultraviolet filters added to the mercury lamp light path for long-term time-lapse	Adaptive Focus Control (AFC, Leica)	Mode: (3) & (4): Super Resolution; (5): Confocal. Frame scanning, Bidirectional scanning. Averaging: (3) & (5) mode: line, number: 2 lines, method: mean; (4) none.
Post-processing	None	None	Deconvolution
Software	Andor Solis (Oxford instruments)	Imaging Solution (Inscoper, SAS)	ZEN blue (Zeiss)

M5- Localisation of ZYG-8 in the C. elegans one-cell embryo

To study ZYG-8 localisation in the zygote, we used *C. elegans* strains displaying a fluorescent labelling of the microtubules (mCherry::tubulin) and a fluorescent labelling of ZYG-8 (mNG::ZYG-8). We analysed deconvolved dual-colour images of fixed embryos obtained by the Zeiss Airyscan 2 microscope. We investigated pixel-wise colocalisation by producing a scatterplot and computed Pearson's correlation coefficient *r* (Figure S1G-H). Statistical significance was assessed through the Costes statistical test using 200 rounds of randomisation and a bin width of 0.001 (Costes et al., 2004). This analysis was performed using Fiji and the JACoP plugin (Bolte and Cordelières, 2006).

M6- Quantification of ZYG-8 level at the worm and embryo scales

To quantify the ZYG-8 level at the worm scale, we performed a Western blot analysis using protein extracts obtained from different *C. elegans* strains (Figure S2A-B). For each experiment, 30 adult hermaphrodites were picked into 200 µL of M9 buffer and snap-frozen in liquid nitrogen, then thawed, mixed with 6.5 µL loading buffer (DTT 0.1 M), and loaded onto a Mini Protean TGX 4–20% Tris-Glicine gel from Bio-Rad (Hercules, CA). Immunoblots were probed using the primary anti-OLLAS antibody (Novus Biologicals NBP1-06713, 1 mg/mL, host: rat) diluted at 1/2000 and the primary anti-tubulin antibody (Sigma-Aldrich T5168, host: mouse) diluted at 1/2000, and then revealed using an HRP (horseradish peroxidase) conjugated secondary antibody (1:20000; Jackson ImmunoResearch, West Grove, PA; host: rat for antiOLLAS & mouse for anti-tubulin). Membrane saturation and all antibody dilutions were made in PBS–Tween 0.2% and 5% non-fat dry milk. Blots were incubated with WesternBright ECL spray (Advansta K-12049-D50) before detecting by chemiluminescence on an Amersham Imager 680 (GeHealthcare, Chicago, IL). Quantification was performed using Fiji (Schindelin et al., 2012).

To complement the first quantification, we analysed deconvolved dual-colour images of embryos carrying two fluorescent tags: mCherry::tubulin and mNG::ZYG-8. For each embryo, we realised, in the same regions of interest (ROI), red and green fluorescence intensity measurements (mean within ROIs) using Fiji: 2 square ROIs (40 x 40 pixels; 1.6 x 1.6 µm) within the spindle (1 per half-spindle), 3 square ROIs (40 x 40 pixels) within the cytoplasm, 5 curvilinear ROIs on astral microtubules using the segmented line tool, and a ROI (40 x 40 pixels) outside the embryo, the latter to measure the background fluorescence. For spindle and cytoplasm measurements, fluorescence quantification was performed on maximum projected images (3 z-sections centred on the spindle mid-plane). After subtracting the background fluorescence (mean intensity in corresponding ROI) from each fluorescence of interest (spindle, cytoplasm and astral microtubule) and for each channel separately, we computed the ratio of ZYG-8 fluorescence over tubulin fluorescence for the three measurements (Figure S2C-D).

M7- Mapping astral microtubules and characterising their local curvatures and their tortuosity

We stained microtubules using an anti-α-tubulin antibody, clone DM1A raised in mouse, Alexa Fluor® 488 conjugate (Sigma-Aldrich 16-232), diluted at 1/500 in PBS-Tween (0.2%) with BSA (1%). It targets several α-tubulin isoforms in various species. Antibody incubation was

performed overnight at 4°C. Slides were then rinsed for 10 minutes in PBS-Tween (0.2%), twice, in a light-free environment. We could then visualise microtubules all along their length. We identified the z-section(s) as the more appropriate for astral microtubule segmentation. For most embryos, we found a single z-section per embryo for which both centrosome asters were visible. For some embryos having the two centrosomes on distinct z-sections, we considered two planes of interest.

Five-step image processing pipeline to map the local curvatures of the astral microtubules (Figure S3A). **(1)** We used the planes above and below the plane of interest to perform an extended depth of field projecting the 3 z-sections into a single image (Figure S3B) (Forster et al., 2004). We thus better visualise microtubules all along their length. **(2)** We applied the *Orientation Filter Transform* (OFT, number of orientations = 20; radius = 5 pixels) since it is effective in enhancing filamentous pattern against noises (Figure S3C) (Sandberg and Brega, 2007; Zhang et al., 2017). **(3)** We then used the interactive machine-learning tool, *Ilastik,* to segment the astral microtubules (Figure S3D). The dedicated paragraph below provides more detailed information about our segmentation. **(4)** We generated the skeleton of the filament traces from the binary segmentation, taking advantage of *SOAX* (Figure S3E). Further information about the use of SOAX is available in a dedicated paragraph below. **(5)** We finally measured the local curvatures of astral microtubules pixel-by-pixel from the skeleton coordinates, enabling mapping of the local curvatures (Figure S3F). For a set of three neighbouring points sampling the curve, the local curvature corresponds to the inverse of the radius of the circle going through the mid-point and best fitting the two others. Local curvatures were computed along microtubules according to the three-point method (Bicek et al., 2007) using a code retrieved on Matlab Central (Kroon, 2011). In a nutshell, microtubules are viewed as parametrised curves; at each point, the *x* and *y* coordinates are represented as second-order polynomials of the curvilinear abscissa passing through the point and its two neighbours. The curvature is then computed with the Frenet-Serret formula.

Ilastik: We initially trained *Ilastik* using the conditions *zyg-8(RNAi)* and its control during metaphase. This involved approximately 10 manual annotations of microtubules across three embryos for each condition to cover various intensities, contrasts and microtubule shapes. We then used this trained model, referred to as #1M (M for metaphase), to segment, in a semi-supervised way, the astral microtubules in metaphase images. Next, we trained the model to segment anaphase images of *zyg-8(RNAi)* treated embryos and their controls. Indeed, denser microtubule networks in anaphase required additional annotations. This led to the upgraded model #1A that we used to segment anaphase images. We then analysed *zyg-8(or484ts)* mutants and untreated embryos in metaphase. The model was further improved by manually annotating three mutants, resulting in model #2M, which we used to segment images of both mutants and untreated embryos in metaphase. We applied a similar process for segmenting anaphase images of mutants and untreated embryos, enhancing model #2M with a few annotations of anaphase mutants to get the model #2A. The table below summarised the *Ilastik* models used to produce the data displayed in the different figures.

***Ilastik* model**	**Used for data displayed in figures**
#1M	2A; 2E & 3B (*zyg-8(RNAi)* and control); S4A-B (only metaphase)
#1A	2B; 2F & 3C (*zyg-8(RNAi)* and control); S4A-B (only anaphase)
#2M	2C; 2E & 3B (*zyg-8(or484ts)* and untreated); S4C-D
#2A	2D; 2F & 3C (*zyg-8(or484ts)* and untreated); S4E-F

SOAX: We extracted the centrelines from the thick filaments utilising SOAX mostly with standard parameters. We made the few specific adjustments highlighted in the table below. These five adjusted parameters were crucial for accurately delineating the curved regions of the microtubules. The settings used ensured that the centrelines faithfully represented the shapes of the microtubules, as confirmed through visual validation. Additionally, manual corrections could be applied at this stage, especially when two microtubules were mistakenly merged into one.

***SOAX* parameter name**	**Parameter value**
Snake Point Spacing	4
Beta	0.01
Stretch Factor (k_str)	0.8
Radial Near (pixels)	8
Radial Far (pixels)	16

Microtubule shape quantification: We analysed only the astral microtubules that were at least 4 μm long after segmentation, as shorter ones often represented fragments. This approach was appropriate for comparing conditions without seeking absolute quantification. We conducted three complementary approaches.

(1) For each z-projected stack, we calculated the normalised histogram of local curvatures with a bin size of 0.05 μm^{-1} (Figure S3G) and averaged these histograms per condition (e.g., Figure 2A, red). The curvatures, expressed in per micrometer, range from 0.025 to 1 μm^{-1}, corresponding to curvature radii from 40 μm to 1 μm, respectively. 40 μm could be interpreted as mostly straight astral microtubules.

(2) We computed the 95th percentile curvature for each microtubule to capture faithfully the highest curvatures and then took the median of the values over the microtubules of each z-projected stack. We finally compared the set of medians between two conditions (e.g., Figure 2E).

(3) We measured the tortuosity for each microtubule, defined as the curvilinear distance divided by the end-to-end distance (Figure 3A). Straight microtubules have tortuosity values near one, while bent microtubules show higher values. Then, we calculated the medians of tortuosity for each z-projected stack and compared the set of medians between two conditions (e.g., Figure 3B).

M8- Simulation using Cytosim

We used *Cytosim* to simulate cytoskeletal filaments (Nedelec and Foethke, 2007). We modified *Cytosim* to allow multiple fibre types to emanate from a single aster and to exert a given external force on solids, fibres or spheres (doi: 10.5281/zenodo.14246919). We conducted a 2D simulation of the first mitosis of the *C. elegans* zygote, focusing on spindle positioning and the astral microtubule network (Figure S5A). The simulation represented the spindle as a rigid rod implemented with static spindle microtubules, excluding any dynamic elements such as motor proteins. The parameters used in the simulation are detailed in Table S2 and the simulation files are provided as supplemental files S1, S2 and S3 with an exemplar video for each microtubule rigidity (Movies S1-S3). Importantly, to mimic cortical asymmetric pulling forces, we applied external forces at the spindle poles: -180 pN at the anterior pole and +300 pN at the posterior pole (Grill et al., 2003). We attached two types of fibres to each solid, which simulated the centrosomes, to differentiate between spindle and astral

microtubules. The astral microtubules were defined as fibres without dynamics at their minus ends and with plus-ends displaying classic dynamic instability. We set their total number to 75, according to our estimated count of astral microtubules longer than about 2 µm in a 2D plane. Their rigidities were varied at 2, 10, or 25 pN·µm², reflecting the range of *in vitro* measurements (Hawkins et al., 2010), with these differences allowing for visible variations in microtubule curvatures. Additionally, these fibres could engage with cortical force generators simulated as hands bound to the cortex.
We measured both the highest curvatures (95th percentile) and the tortuosity of the astral microtubules (Figure S5B-C) after 45 seconds of simulation, following the approach outlined in method M7.
To estimate the durations of fibre cortical contacts, we analysed fibre lengths, end positions, and cortical anchor positions. When microtubules push against the cortex, they grow slowly due to the force-velocity relationship, remaining near the cell cortex. We first tracked fibre lengths over time to identify instances of very low growth rates (under 0.18 µm/s), indicating moments when the fibres were stalled (neither growing nor shrinking). Next, we calculated the distances between fibre end positions and cortical anchor (hand) positions during these stalling events to identify the events whose distance was below 1 µm. Over 45 seconds (beginning 15 seconds after the simulation started to allow for warm-up), we recorded the durations of fibre cortical contacts and constructed the corresponding histogram. We then fitted it with a mono-exponential model (Kozlowski et al., 2007; Sugioka et al., 2018). This analysis yielded a characteristic lifetime for each simulation run (Figure S5D).

M9- Landing assay to study astral microtubule cortical contacts

The entire microtubule lattices were fluorescently labelled using GFP::TBB-2 or mCherry::tubulin to view them in all their states. We used the same approach as the one previously implemented in (Bouvrais et al., 2021) and available on Zenodo (doi: 10.5281/zenodo.4552485); we combined a Kalman filtering (Kalman, 1960) (gain set to 0.5 and initial noise to 0.05) and *u-track* (Jaqaman et al., 2008) whose parameters are listed in the table below. After tracking the microtubule contacts, we could compute the duration histograms of astral microtubule contacts per embryo and averaged them among each condition (e.g., Figure 4A). We then performed the DiLiPop analysis. This statistical analysis of the microtubule cortical contact durations reveals the short-lived and long-lived populations of astral microtubules at the cortex associated with pulling and pushing events, respectively (Bouvrais et al., 2021). By globally fitting the embryo-set duration distributions of a given condition, we obtained the characteristic lifetimes and densities of the cortical pulling and pushing events (e.g., Figure 5CD). Error bars of the lifetimes and densities were obtained by bootstrapping as explained in (Bouvrais et al., 2021). These densities of events are distinct from instantaneous contact density and thus independent of the cortical contact durations (except in the case of anaphase pulling events due to all force generators being involved in pulling).

u-track step	**Parameter name**	**Parameter value**
Detection	Gaussian standard deviation	1.2
	Alpha-value for initial detection of local maxima	0.14* / 0.12**
	Rolling window time-averaging	3
	Iterative Gaussian mixture-model fitting	0
Tracking	Maximum gap to close	5

	Merge split	0
	Minimum length of track segments from the first step	1
Cost function frame-to-frame linking	Flag for linear motion	1
	Allow instantaneous direction reversal	1
	Search radius lower limit	1
	Search radius upper limit	3
	Standard deviation multiplication factor	3
	Nearest neighbour distance calculation	1
	Number of frames for nearest neighbour distance calculation	4
Cost function close gaps	Flag for linear motion	1
	Search radius lower limit	1
	Search radius upper limit	3
	Standard deviation multiplication factor	3
Nearest neighbour distance calculation	Number of frames for nearest neighbour	4
	Penalty for increasing gap length	1.5
	Maximum angle between linear tracks segments	30

* for GFP::TBB-2 labelling; ** for mCherry::tubulin labelling.

M10- Characterisation of astral microtubule growing and nucleation rates

We labelled microtubule plus-ends using EBP-2::mKate2, which form comet-like structures as the microtubules grow. We performed an analysis of the comets adapted from the article by Srayko *et al.* using Fiji (except otherwise cited) (Srayko et al., 2005). We measured microtubule dynamics during metaphase.

To measure the growth rates, we first denoised the movies using Kalman filtering, with the gain set to 0.5 and the initial noise estimate set to 0.05 (Kalman, 1960). Next, we traced a line along the comet trajectory in the pre-processed movie, restricting to comets that remained visible for at least 10 frames (4 s). We generated a kymograph from the traced line to assess comet displacement velocity. We repeated the measurement approximately 50 times for each condition across about ten embryos (about 25 comets per centrosome).

To measure the nucleation rate, we first denoised the movies using Candle filtering (parameters: *beta* = 0.1; *patchRadius* = 1; *searchRadius* = 3; *back* = 1). We did not apply additional pre-processing for *zyg-8(RNAi)* treatment, *zyg-8* overexpression and their control. However, for *zyg-8(or484ts)* mutants and untreated embryos, we enhanced contrast using an unsharp mask (*radius* = 1; *Mask weight* = 0.7). Since centrosomes moved during metaphase, we applied Icy's rigid registration plugin (v1.4) on the denoised images (de Chaumont et al., 2012). This registration aligns each image with a reference image to correct for centrosome displacements, as described in (Soler et al., 2022). Doing so, we accurately counted the comets crossing a half-circle region of interest (ROI) positioned 9 µm from the centrosome after (Srayko et al., 2005). We generated kymographs over 150 frames (60 seconds). To address the inhomogeneous background in the raw kymographs, we applied background subtraction using Gaussian blurring. We then set an intensity threshold to retain the brightest 2% of pixels in *zyg-8(RNAi)* and control kymographs, and 2.5% for *zyg-8* overexpression ones, *zyg-8(or484ts)*, and their controls. Finally, we used the "Analyze particles" command and counted objects considered as individual EBP-2 comets (particle size: 2 pixels to infinity, 0.14 µm/pixel). Measurements were done for both anterior and posterior centrosomes, except when only a single centrosome was clearly visible.

M11- Centrosome-tracking assay and oscillation characterisation

The tracking of labelled centrosomes (e.g., mCherry::TBG-1) and the analysis of their trajectories were conducted using custom tracking software developed using Matlab (The MathWorks) (Pécréaux et al., 2006a). Tracking of γ-tubulin labelled embryos fixed in -20°C methanol demonstrated an accuracy of 10 nm (Pécréaux et al., 2016). To determine embryo orientations and centres, we performed cross-correlation of the embryo background cytoplasmic fluorescence with artificial binary images that represented an embryo. We calculated the embryo size (lengths of the long and short axes) using the active contour algorithm (Pécréaux et al., 2006b). From the spindle pole positions, we measured the maximal amplitudes of anaphase oscillations (peak-to-peak) for both poles, normalised by the short axis of the embryo, as well as their maximum frequencies (e.g., Figure 5A-B). We also assessed the most posterior positions of the posterior centrosome, normalised by the embryo's long axis. The posterior displacement reflects cortical pulling forces (e.g., Figure S8) (Pécréaux et al., 2006a). Finally, we quantified the spindle position (the midpoint between the two centrosomes), and the spindle orientation (the angle between the spindle axis and the embryo's long axis) at the end of mitosis, specifically at 150 s after the anaphase onset (Figure 6C-D).

M12- Centrosome-size measurements

We analysed images of embryos labelled with mCherry::TBG-1 or GFP::TBG-1 by tracking their centrosomes (cf. M11). From the tracked positions, we extracted vignettes of 6.4 x 6.4 µm (40 x 40 pixels). We then performed a 2D Gaussian least-square fit to characterise both the posterior and anterior centrosomes every second (Kolin, 2003). From these fits, we obtained the *x*- and *y*-widths and calculated the diameter of the centrosomes, assuming they are round, using the formula: $D = \sqrt{x^2 + y^2}$.

M13- Fourier analysis of the positional micromovements

We investigated the micromovements of spindle position along the transverse axis over time, based on our previously published method (Pecreaux et al., 2016). From the time series of the spindle position along the transverse axis during anaphase obtained by tracking the poles (cf. M11), we computed the power spectral density (*PSD*) of the signal multiplied by the Hann window to safeguard against artefact due to the position difference between the beginning and end of the considered time interval (Mercat, 2016). It contrasted with (Pécréaux et al., 2016), where we selected only embryos with low drift in their position over time, rather than windowing. The Hann windows appeared to be a sensible choice for fitting the *PSD* later since its discrete Fourier transform has only three non-zero coefficients (harris, 1978; Mercat, 2016). Therefore, once convolved to the real *PSD* of the spindle length, it minimally "blurs" it. We then fitted this *PSD* with a second-order Lorentzian model, as in (Pécréaux et al., 2016). Along a mechanistic line, this model corresponds to a Kelvin-Voigt-with-inertia discrete model corresponding to a Hookean spring (centring, *K*), a dashpot (damping, *G*), and an inertial element (*I*) in parallel. However, these quantities, when retrieved by fitting, depend on the amplitude of the stochastic noise that powers the fluctuations. We, therefore, utilised an alternative representation featuring (1) a diffusion coefficient *D*, inversely proportional to the damping *G* and dependent on the stochastic noise powering the fluctuations (Pécréaux et al., 2016), (2) the centring-to-damping corner frequency f_c ($\propto K/G$) reflecting centring efficiency,

and (3) the damping-to-inertia corner frequency f_0 ($\propto G/I$); the inertia I arising from load-dependent detachment of force generators as for anaphase rocking (Pécréaux et al., 2006a). Such a model was transformed into the Fourier space and convolved on the fly with the transform of the Hann window to perform the fit (Mercat, 2016). We then performed the fit using augmented Lagrangians and refined it using the pattern search algorithm (Audet and Dennis Jr, 2002; Pierre and Lowe, 1975) as implemented in (Mercat, 2016). To quantify the variability from biological origin of the spectrum over N embryos, we estimated the confidence interval at significance 0.05 using bootstrap with bias-corrected and accelerated percentile method (DiCiccio and Efron, 1996; Efron and Tibshirani, 1994). In particular, it involved performing 30 iterations of fitting a set of N embryos drawn with replacement from the available ones. We filtered out 4/30 draws in the case of non-treated paired with *zyg-8* overexpression as the fit resulted in a too-low likelihood.

M14- Statistics

The difference between two samples was assessed by the two-tailed Student's t-test with Welch–Satterthwaite correction for unequal variance. When Gaussian distribution cannot be assumed, we used the Mann–Whitney test (also known as the Wilcoxon rank-sum test). We used it for instance to compare proportions of microtubule curvatures () or microtubule highest curvatures (Figure 2E-F) or microtubule tortuosities (Figure 3B-C). For the sake of simplicity, we reported confidence levels using diamond (◊, $0.01 < p \leq 0.05$), stars (*, $0.001 < p \leq 0.01$; **, $1\text{x}10^{-4} < p \leq 0.001$; ***, $1\text{x}10^{-5} < p \leq 1\text{x}10^{-4}$) or ns (non-significant, $p > 0.05$). We abbreviated the standard deviation by SD. Last, we used the Kolmogorov–Smirnov test to reveal whether the two distributions differed significantly (distributions of contact durations or of local curvatures).

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